Protein Engineering Studies on Structure and Function of Thermolysin, Matriptase, and Hepatocyte Growth Factor Activator Inhibitor Type 1(Digest_要約)

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論 文 の 要 約

1. Title

Protein Engineering Studies on Structure and Function of Thermolysin, Matriptase, and Hepatocyte Growth Factor Activator Inhibitor Type 1

(サーモライシン、マトリプターゼおよび肝細胞増殖因子活性化因子阻害物質タイプ１の構造および機能ドメインの役割)

2. Summary

Protein engineering method is widely applied in enzyme studies to generate novel enzymes and improve the properties of enzymes, such as optimal pH, substrate specificity, thermostability, pH-stability, and catalytic activity, using recombinant DNA technology. This method is also exploited to investigate structure-function relationships of enzymes and related protein inhibitors.

In this thesis, protein engineering method was applied to two proteases and a related protein inhibitor for investigating the functional and structural roles of the domains and amino acid residues in them. One of the two proteases is matriptase, which is a serine protease expressed abundantly in epithelial cells and keratinocyte and plays a key role in the maintenance of epithelial integrity and homeostasis. The other is thermolysin, which is a metalloproteinase from *Bacillus thermoproteolyticus* and one of the most representative industrial enzymes. It has been widely utilized to synthesis of peptides such as a precursor of an artificial sweetener aspartame.

Matriptase (also known as membrane-type serine protease 1, epithin, suppression of tumorigenecity 14, *etc*.) is a type II transmembrane serine protease consisting of

catalytic domain and non-catalytic stem domain. This protease has trypsin-like activity, and is known to cleave to activate a variety of proteins, including single-chain urokinase-type plasminogen activator (sc-uPA), pro-hepatocyte growth factor (HGF), and the precursor form of prostasin, a glycosyl-phosphatidylinositol-linked serine protease. These characteristics, together with the abundant expression in surface-lining epithelial cells such as enterocytes, lead to a proposal that matriptase is a key upstream regulator of the epithelial-cell turnover, including proliferation, migration, differentiation, and exfoliation.

The activity of matriptase is strictly regulated through the inhibition by a cognate Kunitz-type inhibitor, hepatocyte growth factor activator inhibitor type 1 (HAI-1). HAI-1 is an epithelial-derived, serine protease inhibitor with multiple domains including two protease-inhibiting Kunitz domains. HAI-1 was isolated originally from the conditioned medium of a human stomach carcinoma MKN45 cell line as a potent inhibitor of HGF activator, a 53-kDa serine protease responsible for proteolytic activation of the inactive single chain precursor of HGF (pro-HGF). HAI-1 is believed to play a crucial role in growth factor-mediated biological processes such as tissue regeneration by counteracting the HGF activator activity. HAI-1 is produced first as a type I membrane protein with a molecular mass of 66 kDa. The extracellular domain can be released by cleavage with certain proteases. At least two HAI-1 species of 58 and 40 kDa are found in conditioned media of MKN45 cells and transfected COS-1 cells. The 58-kDa species (58-kDa HAI-1) has all of the HAI-1 extracellular region and comprises the N-terminal domain with eight cysteine residues, followed by the internal domain rich in acidic amino acid residues, the first Kunitz domain (Kunitz domain I), the low-density lipoprotein receptor A module (LDLRA)-like domain (LDLRA domain),

and the second Kunitz domain (Kunitz domain II). The 40-kDa species (40-kDa HAI-1) lacks Kunitz domain II, but is thought to be responsible for inhibiting target proteases *in vivo* because it exhibits higher inhibitory activity against HGF activator than does 58-kDa HAI-1. The ratio in the amounts of matriptase and HAI-1 has been shown to increase in late-stage tumors. This imbalance might lead to the excessive activity of matriptase and would be significant for the development of advanced disease. Elucidation of the mechanism by which HAI-1 inhibits matriptase is considered important for obtaining strategies to retard cancer progression. In Chapter 1, the roles of the functional and structural domains of HAI-1 in the inhibition of matriptase were investigated by producing HAI-1 mutants made up with various combinations of domains and comparing their inhibitory activity against matriptase. In Chapter 2, the roles of the stem domain of matriptase in the interaction with HAI-1 were addressed by producing matriptase variants lacking the stem domain.

Thermolysin [EC 3.4.24.27] is a thermophilic and halophilic neutral metalloproteinase produced in the culture broth of *Bacillus thermoproteolyticus*. Its amino acid sequence and crystal structures have been determined. It has been widely utilized to synthesis of peptides such as a precursor of an artificial sweetener aspartame, and thus is one of the most representative industrial enzymes. The improvement of its activity and stability are important subjects. In Chapter 3, to further stabilize thermolysin, the effect of the amino acid residue located at its C-terminal domain on the stability of thermolysin was examined by producing fourteen Val315 variants. In Chapter 4, to understand the structural determinants of substrate specificity of thermolysin, the role of the amino acid residue located in the S_1' subsite was examined by producing nineteen Leu202 variants. Summary of each chapter is described below.

Chapter 1 Roles of Functional and Structural Domains of Hepatocyte Growth Factor Activator Inhibitor Type 1 in the Inhibition of Matriptase

Hepatocyte growth factor activator inhibitor type 1 (HAI-1) inhibits serine proteases that have potent pro-hepatocyte growth factor-converting activity, such as the membrane-type serine protease, matriptase. HAI-1 comprises an N-terminal domain, followed by an internal domain, first protease inhibitory domain (Kunitz domain I), low-density lipoprotein receptor A module (LDLRA) domain, and a second Kunitz domain (Kunitz domain II) in the extracellular region. The roles of these domains in the inhibition of matriptase were assessed. Soluble forms of recombinant rat HAI-1 mutants made up with various combinations of domains were produced, and their inhibitory activities toward the hydrolysis of a chromogenic substrate were analyzed using a soluble recombinant rat matriptase. Kunitz domain I exhibited inhibitory activity against matriptase, but Kunitz domain II did not. The N-terminal domain and Kunitz domain II decreased the association rate between Kunitz domain I and matriptase, whereas the internal domain increased this rate. The LDLRA domain suppressed the dissociation of the Kunitz domain I-matriptase complex. Surprisingly, an HAI-1 mutant lacking the N-terminal domain and Kunitz domain II showed an inhibitor constant of 1.6 pM, and the inhibitory activity was 400 times higher in this HAI-1mutant than in the mutant with all domains. These findings, together with the known occurrence of an HAI-1 species lacking the N-terminal domain and Kunitz domain II in *vivo*, suggest that the domain structure of HAI-1 is organized in a way that allows HAI-1 to flexibly control matriptase activity.

Chapter 2 Role of the Stem Domain of Matriptase in the Interaction with Its Physiological Inhibitor, Hepatocyte Growth Factor Activator Inhibitor Type 1

Matriptase contains the non-catalytic domains (stem domain) and catalytic domain in the extracellular region. The role of the stem domain in the interaction between matriptase and HAI-1 was addressed. Secreted variants of recombinant matriptase containing the entire extracellular domain (HL-matriptase) or only the catalytic domain (L-matriptase) were prepared, and the inhibition activities of a cell membrane-anchored form of recombinant HAI-1 (maHAI-1) against the matriptase variants in the hydrolysis of peptidyl-4-methyl-coumaryl-7-amide (MCA) substrates were compared. HL-matriptase and L-matriptase were inhibited by purified maHAI-1 with a similar extent when *t*-butyloxycarbonyl (Boc)-Gln-Ala-Arg-MCA (substrate 1) and acetyl-Lys-Thr-Lys-Gln-Leu-Arg-MCA (substrate 2) were used as substrates. However, HL-matriptase was inhibited more strongly than L-matriptase by maHAI-1 in the hydrolysis of Boc-[(2*S*)-2-amino-3-(benzyloxycarbonyl)propionyl]-Pro-Arg-MCA (substrate 3). These results show that the stem domain of matriptase facilitates the inhibitory interaction of this protease with maHAI-1 in the hydrolysis of substrate 3, although it has no effect in the hydrolysis of substrates 1 and 2. To my knowledge, this is the first evidence that the stem domain of matriptase can affect the interaction between this protease and HAI-1.

Chapter 3 Involvement of Val315 Located in the C-terminal Region of Thermolysin in Its Expression in *Escherichia coli* and Its Thermal Stability

Thermolysin is a thermophilic and halophilic zinc metalloproteinase that consists of β-rich N-terminal (residues 1–157) and α-rich C-terminal (residues 158–316) domains. Expression of thermolysin variants truncated from the C-terminus was examined in *E. coli* culture. The C-terminal Lys316 residue was not significant in the expression, but Val315 was critical. Variants in which Val315 was substituted with fourteen amino acids were prepared. The variants substituted with hydrophobic amino acids such as Leu and Ile were almost the same as wild-type thermolysin (WT) in the expression amount, α-helix content, and stability. Variants with charged (Asp, Glu, Lys, and Arg), bulky (Trp), or small (Gly) amino acids were lower in these characteristics than WT. All variants exhibited considerably high activities (50–100% of WT) in hydrolyzing protein and peptide substrates. The expression amount, helix content, and stability of variants showed good correlation with hydropathy indexes of the amino acids substituted for Val315. Crystallographic study of thermolysin has indicated that Val315 is a member of the C-terminal hydrophobic cluster. The results obtained in this study indicate that stabilization of the cluster increases thermolysin stability and that the variants with higher stability are expressed more in the culture. Although thermolysin activity was not severely affected by the variation at position 315, the stability and specificity were modified significantly, suggesting the long-range interaction between the C-terminal region and active site.

Chapter 4 Role of Leu202, a Residue Located at the S_1' Subsite of Thermolysin, on Its Substrate Specificity

The role of Leu202 in thermolysin, a residue located in the S_1' subsite, was examined using 19 single variants at position 202 and measuring their hydrolysis activities for casein, *N*-[3-(2-furyl)acryloyl]-glycyl-L-leucine amide (FAGLA), *N*-carbobenzoxy-L-aspartyl-L-phenylalanine methyl ester (ZDFM), and *N*-[3-(2-furyl)acryloyl]-L-alanyl-L-phenylalanine amide (FAAFA) at pH 7.5 at 25°C. All variants except for the variant L202W, in which Leu202 was replaced to Trp, retained activities for all substrates. The L202W variant lacked the activity for ZDFM and retained for the other three. The correlation between the activities of variants for FAGLA and ZDFM was weak with the correlation coefficient (r) of 0.40, whereas that between the activities for FAGLA and FAAFA was fairly good (r = 0.87). These correlations were marked in the variants L202K and L202R: the k_{cat}/K_m values of L202K and L202R for FAGLA were 60 and 30%, respectively, of that of wild-type thermolysin, while those for ZDFM were 370 and 360%, and those for FAAFA were 330 and 170%. These results suggest that Leu202 is not much critical for catalytic activity but plays an important role in substrate specificity of thermolysin.